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RESEARCH ARTICLE

Biosynthetic and Genetic Relationships of B-series Fumonisins Produced by *Gibberella fujikuroi* Mating Population A†

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ABSTRACT Fumonisins are mycotoxins produced by the maize pathogen *Gibberella fujikuroi* mating population A and frequently contaminate maize. Wild-type *G. fujikuroi* produces four B-series fumonisins, FB_1, FB_2, FB_3 and FB_4. These toxins are identical in structure except for the number and positions of hydroxyls along their linear carbon backbone. To elucidate the genetic and biosynthetic relationships among these fumonisins, we conducted meiotic and biochemical analyses of *G. fujikuroi* mutants with altered fumonisin production that resulted from defective alleles at three loci, *Fum1*, *Fum2* and *Fum3*. These mutants produced either no fumonisins, only FB_2 and FB_4, or only FB_3 and FB_4. Genetic analyses revealed the orientation of the *Fum* loci along linkage group 1 of the fungus. The mutants were grown together in pair-wise combinations to determine if their fumonisin production phenotypes could be complemented. When FB_3- and FB_2-producing mutants were grown together, complementation occurred. However, when a nonproducing mutant was grown with a FB_2- or FB_3-producing mutant, complementation did not occur or was incomplete. When purified FB_2, FB_3, or FB_4 was fed to mutant cultures, FB_4 was converted primarily to FB_2, FB_3 was converted to FB_1 and FB_2 was not converted. The results from these assays suggest a previously unrecognized branch in the fumonisin biosynthetic pathway. Published in 1999 by John Wiley & Sons, Ltd.

Key words: *Gibberella fujikuroi*; *Fusarium verticillioides*; fumonisin; mycotoxin biosynthesis

INTRODUCTION

Fumonisins are mycotoxins that are frequently present in maize kernels. These toxins are produced by a number of fungi within the *Gibberella fujikuroi* species complex, but production by *G. fujikuroi* mating population A (MP-A) (anamorph *Fusarium verticillioides*, syn. *F. moniliforme*) is of particular concern, because this fungus is a common ear rot pathogen of maize and is often present in healthy maize tissue (Munkvold and Desjardins,1999). Fumonisins are of concern because they are associated with several animal mycotoxicoses, including leucoencephalomalacia in horses, pulmonary oedema in swine and liver cancer in rats (Nelson *et al.*, 1993). There is also an epidemiological correlation between the consumption of fumonisin-contaminated maize and human oesophageal cancer in some areas of the world where maize is a dietary staple (Marasas, 1996). Fumonisins are structurally similar to the sphingolipid intermediate sphinganine and disrupt sphingolipid metabolism by inhibiting the enzyme sphinganine *N*-acyltransferase. This disruption may in turn be responsible for some fumonisin-induced toxicoses (Wang *et al.*, 1991).

The four B-series fumonisins, which are abbreviated as FB_1, FB_2, FB_3 and FB_4, are generally the most abundant fumonisins present in maize that is naturally infected with *G. fujikuroi* MP-A. Typically, FB_1 makes up about 70% and FB_2 and FB_3 each make up 10–20% of the total fumonisins present in infected maize (Nelson *et al.*, 1993). These fumonisins consist of a linear 20-carbon backbone with an amino group at carbon atom 2 (C-2),

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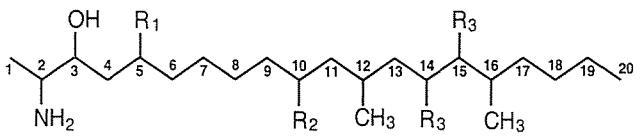

	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH
Fumonisin B_4	H	H

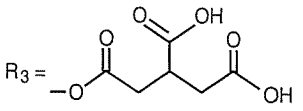

Figure 1. Structure of B-series fumonisins.

tricarballylic esters at C-14 and C-15, and methyl groups at C-12 and C-16 (Figure 1). B-series fumonisins differ from one another only by the presence/absence of hydroxyl groups at the C-5 and C-10 positions of the backbone. Two fumonisin biosynthetic loci (*Fum2* and *Fum3*) responsible for hydroxylation of these positions have been identified. *G. fujikuroi* MP-A strains with a defective *Fum2* allele are unable to hydroxylate the C-10 position, and as a result produce only FB_2 and FB_4, while strains with a defective *Fum3* allele are unable to hydroxylate the C-5 position, and as a result produce only FB_3 and FB_4 (Desjardins *et al.*, 1996a). Meiotic analyses of strains with these defective alleles indicated that *Fum2* and *Fum3* are linked to one another and to a third locus, *Fum1*, which governs whether or not fumonisins are produced (Desjardins *et al.*, 1992).

Although understanding of the fumonisin biosynthetic pathway is limited, what is known about this process has resulted from a combination of biochemical, genetic and molecular genetic studies. Several lines of evidence indicate that C-3 to C-20 of the fumonisin backbone are a product of polyketide synthesis (Blackwell *et al.*, 1994; Proctor *et al.*, 1999) and that condensation of the putative polyketide with alanine gives rise to the C-2 amino group as well as C-1 and C-2 of the backbone (Branham and Plattner, 1993). In addition, there is evidence that oxygen atoms attached directly to the backbone are derived from molecular oxygen (Caldas *et al.*, 1998). In general, fungal toxin biosynthesis proceeds from simple molecules to more complex molecules. This increasing complexity often includes multiple oxygenations, as in the cases of trichothecene and patulin biosynthesis (Desjardins *et al.*, 1993; Betina, 1989). Based on this precedence, the structural relationship of B-series fumonisins, and the genetic analysis of mutants with defective *Fum2* and *Fum3* alleles, Desjardins *et al.*, (1996a) proposed a pathway for the last steps of fumonisin biosynthesis. In the proposed pathway, FB_4 was the immediate precursor of both FB_2 and FB_3, which were formed via hydroxylation of the FB_4 backbone at C-5 and C-10, respectively. FB_2 and FB_3 were in turn immediate precursors of FB_1, which was formed via C-10 hydroxylation of FB_2 and C-5 hydroxylation of FB_3. Thus, the biosynthesis of fumonisins was proposed to proceed from the least oxygenated homologue, FB_4, to the most oxygenated homologue, FB_1 (Desjardins *et al.*, 1996a). In contrast, Blackwell *et al.* (1994) suggested that FB_2 and FB_3 were cometabolites rather than precursors of FB_1.

In this study, we carried out biochemical and genetic analyses of strains of *G. fujikuroi* MP-A with altered fumonisin production phenotypes in order to further elucidate the fumonisin biosynthetic pathway. The biochemical analysis revealed a branch in the pathway for B-series fumonisin biosynthesis that was not apparent from the structural relationships of these compounds. In addition, the genetic analysis facilitated the mapping of three fumonisin biosynthetic loci along a *G. fujikuroi* MP-A chromosome.

MATERIALS AND METHODS

Strains and Media

Strains of *G. fujikuroi* MP-A used in this study, their fumonisin production phenotypes and their genotypes with respect to *Fum* loci are shown in Table 1. Genotype designations for these strains are based on the proposed genetic nomenclature for plant pathogenic fungi (Yoder

Table 1. Strains of *G. fujikuroi* MP-A used in this study

Strain	Fumonisin production	Proposed genotype	RAPD markers	Reference
M-3120	FB_1, FB_2, FB_3, FB_4	*Fum1-1, Fum2-1, Fum3-1*	A16−, H3−	Leslie, 1991; Desjardins *et al.*, 1996a
M-3125	FB_1, FB_2, FB_3, FB_4	*Fum1-1, Fum2-1, Fum3-1*	A16+, H3−	Leslie, 1991; Desjardins *et al.*, 1996a
397-R-36	FB_3, FB_4	*Fum1-1, Fum2-1, Fum3-2*	ND	Desjardins *et al.*, 1996a
109-R-7	FB_2, FB_4	*Fum1-1, Fum2-2, Fum3-1*	ND	Desjardins *et al.*, 1996a
510-R-20	FB_2, FB_4	*Fum1-1, Fum2-2, Fum3-1*	A16+, H3+	Desjardins *et al.*, 1996a
57-7-7	None	*Fum1-2, Fum2-1, Fum3-1*	ND	Desjardins *et al.*, 1992
UV26	FB_3, FB_4	*Fum1-1, Fum2-1, Fum3-3*	A16−, H3−	This study
575-R-5	FB_3, FB_4	*Fum1-1, Fum2-1, Fum3-3*	A16−, H3−	This study

ND, not determined.

et al., 1986). Media employed in this study were GYAM, GYP (2% glucose, 1% peptone and 0.3% yeast extract) (Hohn and Desjardins, 1992), V-8 Juice agar (Tuite, 1969) and cracked maize kernels (Leslie *et al.*, 1992). GYAM medium was a modification of the medium described by Clouse *et al.* (1985) and was prepared as described previously (Proctor *et al.*, 1999). Nitrogen non-utilizing mutants were generated and used in vegetative compatibility assays as previously described (Klittich and Leslie, 1988).

Fumonisin Assays

Purified FB_1, FB_2, FB_3 and FB_4 for use as HPLC standards and in feeding studies were obtained as described previously (Poling and Plattner, 1996; Poling and Plattner, 1999). For analysis of fumonisin production by *G. fujikuroi* MP-A, we used cracked maize kernel (Plattner *et al.*, 1996) or GYAM cultures (Proctor *et al.*, 1999) of the fungus and HPLC of OPA-derivatized samples or HPLC-mass spectrometry (LC/MS) of underivatized samples (Desjardins *et al.*, 1994; Plattner *et al.*, 1996). Mixed strain cultures were grown in 10 g cracked maize or 20 ml liquid GYAM cultures and inoculated with mycelial plugs from V-8 Juice agar cultures (Proctor *et al.*, 1999). In fumonisins feeding experiments, single strain cultures were inoculated with 5×10^5 conidia per ml liquid GYAM and incubated at 28 °C for 5 days at 200 rev min^{-1}. FB_2, FB_3 and FB_4 were dissolved in methanol at 10 mg ml^{-1}. Individual fumonisins were added to cultures by first dispensing 0.1 ml (for 10 ml culture) or 1 ml (for 100 ml culture) of methanol solution, which contained either FB_2, FB_3 or FB_4, into an Erlenmeyer flask of the appropriate size and placed in a sterile biosafety hood to allow the methanol to evaporate. Once the methanol had evaporated, 5-day-old liquid GYAM culture was added to each flask. The cultures were incubated for a further 9 days and then analysed by LC/MS for their fumonisin content.

Genetic Manipulations

For mutagenesis, conidia of strain M-3120 were harvested from 5- to 7-day-old V-8 Juice agar cultures by suspending them in water and filtering through sterile muslin. Fifteen milliliters of a 5×10^5 conidia ml^{-1} solution was dispensed into a petri plate on a rotary shaker set at 80 rev min^{-1}. Conidia were exposed to ultraviolet (UV) radiation from a Spectroline UV lamp elevated 12 cm above the shaker. Individual colonies arising from conidia that survived irradiation for 80 to 100 s (1–2% survival) were examined for fumonisin production in GYAM culture as described above. Genetic analyses were done by mating *G. fujikuroi* strains on carrot agar and recovering individual random ascospores (Desjardins *et al.*, 1996a).

Nucleic Acid Manipulations

Genomic DNA was isolated from *G. fujikuroi* strains using a previously described method (Desjardins *et al.*, 1996a). The RAPD primers used in this study were Operon oligonucleotides OPA16 (AGCCAGCGAA) and OPH3 (AGACGTCCAC). The RAPD marker amplified with OPA16 was a 0.75 kb fragment and the OPH3 marker was a 1.7 kb fragment (Desjardins *et al.*, 1996b). RAPD PCR was carried out in a Perkin-Elmer 480 thermocycler essentially as described by Williams *et al.* (1990) except 25–75 ng of genomic DNA and 0.225 mM of each dNTP per were used. Thermocycler settings for RAPD PCR were as follows: 35 cycles of 94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min. PCR products were analysed via agarose gel electrophoresis following standard protocols (Sambrook *et al.*, 1989).

RESULTS

Genetic Analysis of Fumonisin Production Mutants

G. fujikuroi MP-A strain UV26 was derived from a conidium that survived UV irradiation. This strain produced 108–448 μg g^{-1} FB_3 in cracked maize culture but no detectable FB_1 or FB_2. Although FB_4 production by strain UV26 was not rigorously quantified, the presence of FB_4 in culture extracts of this strain was confirmed by LC/MS analysis. FB_3 and FB_4 both lack the C-5 hydroxyl group that is present in FB_1 and FB_2. Since the C-5 hydroxyl is the only structural feature that is absent in FB_4 and FB_3 but present in FB_1 and FB_2, mutant strain UV26 must lack the ability to hydroxylate the C-5 position of the fumonisin backbone.

Strain UV26 was crossed to three other *G. fujikuroi* MP-A strains to determine whether its FB_3 production phenotype was conferred by a mutation at a single locus and, if so, whether this locus was linked to other previously identified *Fum* loci. Unless stated otherwise, the production of FB_4 by progeny from these crosses was not determined. In cross 575, strain UV26 was crossed with strain M-3125, which produces the wild-type complement of FB_1, FB_2, FB_3, and FB_4. The segregation ratio of 9 FB_3-producing and 10 wild-type progeny from this cross did not differ significantly from the 1:1 ratio expected if the UV26 phenotype resulted from a mutation at a single locus or two or more closely linked loci.

In cross 636, strain 575-R-5, an FB_3-producing progeny of strain UV26 (from cross 575 above), was crossed with another FB_3-producing strain, 478-R-3. Strain 478-R-3 carries the mutant *Fum3-2* (previously *fum3*$^-$) allele, which originated in a natural variant of *G.*

Table 2. Fumonisin production in mixed cultures of *G. fujikuroi* MP-A strains with different fumonisin production phenotypes

1st Strain	2nd Strain	FB_1[a]	FB_2[a]	FB_3[a]
M-3125	None	156	24	20
575-R-5	None	0	0	607
397-R-36	None	0	0	449
109-R-7	None	tr	302	0
510-R-20	None	tr	8	0
57-7-7	None	0	0	0
575-R-5	109-R-7	26	95	404
575-R-5	510-R-20	3	2	6
575-R-5	397-R-36	0	0	352
575-R-5	57-7-7	14	0	222
397-R-36	109-R-7	20	57	275
397-R-36	510-R-20	21	25	71
397-R-36	57-7-7	20	0	207
109-R-7	57-7-7	2	69	1
109-R-7	510-R-20	1	22	0
510-R-20	57-7-7	1	6	tr

[a] Values are μg fumonisin per ml liquid GYAM culture.
tr: indicates the given fumonisin was present but its concentration was below the level of quantification.

fujikuroi and confers the FB_3 production phenotype (Desjardins *et al.*, 1996a). All 90 progeny from cross 636 that were examined produced FB_3, but no FB_1 or FB_2. This apparent lack of recombination between *Fum3-2* and the UV26 mutation suggests that these two loci are allelic or closely linked. The fact that strains carrying *Fum3-2* and those carrying the UV26 mutation have identical phenotypes (i.e. the inability to hydroxylate C-5 of the fumonisin backbone) is a further indication that the two loci are allelic. Therefore, we will hereafter refer to the locus containing the UV26 mutation as the *Fum3-3* allele of *Fum3*.

In cross 648, strain 575-R-5 was crossed with strain 510-R-20 to examine the linkage of the *Fum2* and *Fum3* loci. Strain 510-R-20 has the *Fum2-2* allele (previously *fum2*$^-$) at the *Fum2* locus, which governs C-10 hydroxylation of the fumonisin backbone. Strains with the *Fum2-2* allele lack the ability to hydroxylate C-10 and as a result produce FB_2 and FB_4 but not FB_1 or FB_3 (Desjardins *et al.*, 1996a). Their respective phenotypes indicate that 510-R-20 has the wild-type *Fum3* allele, *Fum3-1*, while 575-R-5 has the wild-type *Fum2* allele, *Fum2-1*. Of the 239 single ascospore progeny from cross 648 that were examined, 120 produced FB_2 and 119 produced FB_3. Recombination between *Fum2* and *Fum3* should have resulted in FB_1-producing progeny with the *Fum2-1/Fum3-1* genotype and or nonproducing progeny (no FB_1, FB_2 or FB_3 produced) with the *Fum2-2/Fum3-3* genotype. Since such progeny were not recovered from cross 648, recombination between the *Fum2* and *Fum3* loci must not have occurred, indicating the two loci are tightly linked.

We also examined a subset of 96 progeny from cross 648 for recombination between the *Fum* loci and two RAPD markers, OPA16 and OPH3. The RAPD markers were previously identified during a bulked segregant analysis of *Fum1* alleles (Desjardins *et al.*, 1996b). Analysis of parent strains 510-R-20 and 575-R-5 revealed that both RAPD markers were present in strain 510-R-20 but absent in strain 575-R-5. Analysis of the progeny for the RAPD markers revealed that the frequency of recombination between *Fum2/Fum3* and OPA16 was 12.5% and between *Fum2/Fum3* and OPH3 was 23.9%.

Complementation of Fumonisin Production in Mixed Cultures

To determine whether strains of *G. fujikuroi* MP-A with different fumonisin production phenotypes could complement one another, we grew strains with these different phenotypes together in mixed cultures. Initially, FB_2-producing strain 510-R-20 (*Fum2-2,Fum3-1*) was grown with FB_3-producing strain 575-R-5 (*Fum2-1,Fum3-3*) in cracked maize cultures. In these cultures, FB_1 accumulated along with FB_2 and FB_3 indicating that complementation occurred. The concentrations of FB_1, FB_2 and FB_3 in these cultures were 163, 289 and 698 μg g^{-1} cracked maize, respectively. Using *nit* mutants, we determined that strains 575-R-5 and 510-R-20 were not vegetatively compatible. Therefore, the apparent complementation of fumonisin production phenotypes was not dependent on the vegetative compatibility of the strains.

Based on the above results, we did a series of experiments in which FB_3-producing, FB_2-producing, and nonproducing strains were grown in pair-wise combinations in liquid GYAM. Fumonisin production

Table 3. Fumonisin production in liquid cultures of *G. fujikuroi* MP-A strains to which FB_2, FB_3 and FB_4 were added.

Strain	Fumonisin added	Per cent fumonisin recovered[a]		
		FB_1	FB_2	FB_3
None	FB_2	0	100	0
	FB_3	0	0	100
	FB_4	0	0	0
M-3125	None	78	12	10
	$FB_2 + FB_3$	27	53	20
575-R-5	None	0	0	100
	FB_2	0	41	59
	FB_4	0	0	100
397-R-36	None	0	0	99
	FB_2	0	51	49
109-R-7	None	0	100	0
	FB_3	7	86	36
	FB_4	0	100	0
510-R-20	None	0	100	0
	FB_3	47	tr	52
57-7-7	None	0	0	0
	FB_2	0	100	0
	FB_3	20	0	80
	FB_4	0	95	5

[a] Values are the per cent of each fumonisin recovered relative to the combined concentration of FB_1, FB_2, and FB_3.
tr: indicates the given fumonisin was present but its concentration was below the level of quantification. The final concentration of fumonisins in the medium immediately following mixing of cultures and fumonisins was approximately 100 $\mu g\ ml^{-1}$.

in the mixed liquid cultures of FB_3-producing strain 575-R-5 and FB_2-producing strain 510-R-20 was qualitatively the same as in mixed cracked maize cultures of these two strains in that FB_1, FB_2 and FB_3 accumulated (Table 2). However, the fumonisin levels were much lower in liquid cultures than in cracked maize cultures. The complementation of production phenotypes was also observed when 575-R-5 was grown in mixed culture with FB_2-producing strain 109-R-7 and when FB_3-producing strain 397-R-36 was grown with either FB_2-producing strain (109-R-7 or 510-R-20). Mixed cultures of either FB_3-producing strain (397-R-36 or 575-R-5) and a nonproducing strain (57-7-7) produced FB_1 and FB_3, but not FB_2 (Table 2). Mixed cultures of either FB_2-producing strain (109-R-7 and 510-R-20) and the nonproducing strain consistently produced FB_2 at moderate levels, low or trace amounts of FB_3 and low levels (1–2 $\mu g\ ml^{-1}$) of FB_1. However, similarly low levels of FB_1 also accumulated when the FB_2 producing strains were grown alone or with one another (Table 2).

Conversion of Exogenous Fumonisins by *G. fujikuroi*

The fact that vegetative incompatibility did not prevent complementation of the fumonisin production phenotypes of some mutants suggested that complementation resulted from cross-feeding of fumonisins, or their precursors, between strains rather than from genetic complementation via hyphal fusion. If this were the case, *G. fujikuroi* MP-A may be able to convert exogenously added fumonisins to other fumonisins. To test this hypothesis, we fed purified FB_2, FB_3 or FB_4 to single strain cultures of *G. fujikuroi* MP-A grown in liquid GYAM. Changes in the types of fumonisin homologues produced when fumonisins were added to the cultures were considered evidence for the conversion of the added fumonisin. In general, the addition of FB_2 or FB_3 to fumonisin-producing strains caused a reduction in production of the toxins. For example, when FB_2 was added to cultures of FB_3-producing strains, FB_3 production decreased by 53 to 59%. Similarly, when FB_3 was added to cultures of a FB_2-producing strains, FB_2 production was reduced by 24 to >90%. Qualitative changes in fumonisin production in the feeding experiments (Table 3) indicate that the nonproducing strain was able to convert FB_4 primarily to FB_2 (95%) and to a much lesser extent to FB_3 (5%). This strain was also able to convert FB_3 to FB_1 but was unable to convert FB_2 to other fumonisins. The FB_2-producing strains were also able to convert FB_3 to FB_1, but FB_3-producing strains were unable to convert FB_2. The addition of FB_4 to cultures of either FB_2- or FB_3-producing strains did not result in changes in the types of fumonisin homologues produced. This result was expected since these strains produce low levels of FB_4. To summarize the feeding experiment results, FB_4 was

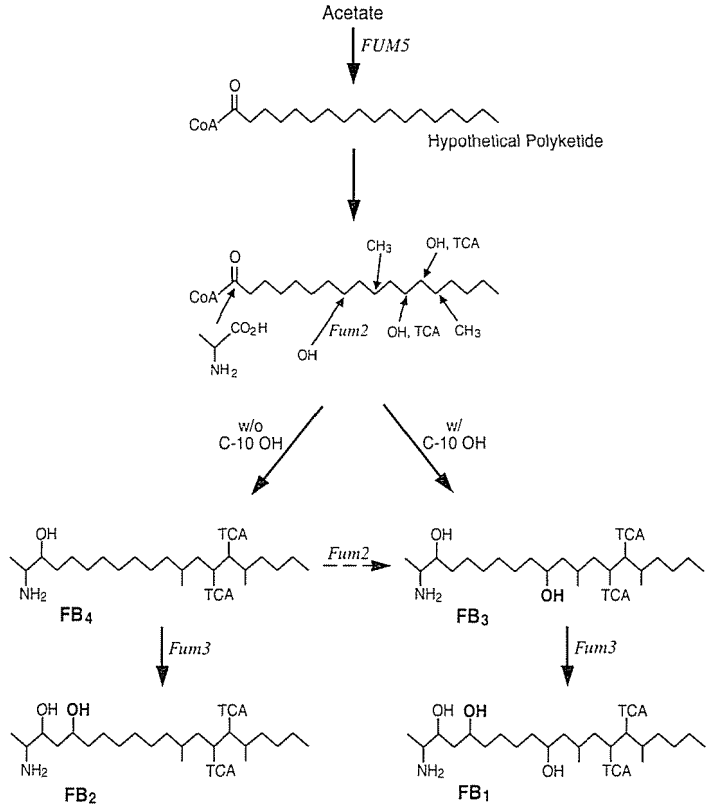

Figure 2. Proposed pathway for the biosynthesis of B-series fumonisins. The order of the C-10 and C-14 methylation, C-12 and C-13 hydroxylation-TCA esterification, condensation with alanine, and carbonyl reduction of the polyketide is not known. The position of the branch point (hydroxylation at C-8 or C-10, depending on whether condensation with alanine has already occurred) relative to these other reactions is also not known. The broken arrow between FB_4 and FB_3 indicates that the conversion of FB_4 to FB_3 was poor (5%) relative to the conversion of FB_4 to FB_2 (95%) TCA indicates tricaballylic acid (see Figure 1)

converted primarily to FB_2, FB_3 was converted to FB_1, and FB_2 was not converted to FB_1.

DISCUSSION

Structural relationships of B-series fumonisins and their relative abundance in wild-type and mutant cultures of *G. fujikuroi* MP-A are consistent with the hypothesis that the biosynthesis of these compounds proceeds from the less hydroxylated to more hydroxylated homologues (Desjardins *et al.*, 1996a). Results from the current study, however, indicate that some steps in the previously hypothesized fumonisin biosynthetic pathway may not occur or may be less significant than originally proposed. In the fumonisin feeding experiments in this study, FB_2 was not converted to other fumonisins. Assuming the fate of exogenously added fumonisins was the same as that of fumonisins produced endogenously by cultures of *G. fujikuroi* MP-A, these results indicate that FB_2 is not a direct precursor of FB_1. Since exogenously added FB_4 was converted primarily to FB_2, it is likely that FB_4 is not a major precursor of FB_3. On the other hand, since FB_3 was converted to FB_1, it is likely that the FB_3 is a direct precursor of FB_1. The results of complementation assays were consistent with the feeding studies. For example, when FB_2 producers were grown in mixed culture with the nonproducer, no FB_1 was produced. However, when an FB_3 producer was grown with either a nonproducer or FB_2 producer, FB_1 was produced. Presumably, the FB_3 producer supplied FB_3, which was taken up and converted to FB_1 by the other strains in these mixed cultures.

The results of the feeding and complementation assays reported here are consistent with the revised fumonisin biosynthetic pathway presented in Figure 2. This pathway is similar to the previously proposed pathway but branches at some point prior to the formation of FB_4. One branch of the pathway leads to the formation of FB_2

via FB_4, whereas the other branch leads to FB_1 via FB_3. Since about 5% of the exogenously added FB_4 was converted to FB_3, it is likely that there is some cross-feeding from one branch to the other. The fact that FB_1 is the most abundant fumonisin produced by wild-type strains of *G. fujikuroi* MP-A suggests that the branch leading to FB_1 is the more active pathway.

Prior to the formation of FB_3 and FB_4, the putative 18-carbon polyketide that forms most of the fumonisin backbone undergoes methylation at C-10 and C-14, hydroxylation and esterification at C-12 and C-13, condensation with alanine at C-1 and carbonyl (C-1) reduction. The order of these reactions in the fumonisin biosynthetic pathway is not known, except that the hydroxylation of C-12 and C-13 has to occur before esterification of these positions. Furthermore, the position of the pathway branch point (i.e. C-10 hydroxylation) relative to these other reactions is not known. Since these other five reactions should result in the formation of FB_4, the branch point must occur prior to at least one of the reactions. In addition, since a small percentage of FB_4 can undergo C-10 hydroxylation, the primary substrate for C-10 hydroxylation may be highly similar in structure to FB_4.

The formation of FB_1 and FB_2 via different branches of the fumonisin biosynthetic pathway is reminiscent of the two branches of the aflatoxin pathway that lead to the formation of aflatoxins B1 and B2 (AFB1 and AFB2, respectively). These two aflatoxins are identical in structure except for a single functional group: a carbon-carbon double bond in AFB1 is absent in AFB2. With such a minor structural difference between these two aflatoxins, researchers initially proposed that AFB2 was formed directly from AFB1 via saturation of the double bond (Heathcote *et al.*, 1976). However, these two compounds are formed via different, parallel branches in the aflatoxin biosynthetic pathway (Bhatnagar *et al.*, 1991; Brown *et al.*, 1999).

Strains of *G. fujikuroi* MP-A with variant fumonisin production phenotypes have contributed to the understanding of fumonisin biosynthesis. These strains have been recovered by screening large numbers of field isolates (Desjardins *et al.*, 1992; Desjardins *et al.*, 1996a) and by the molecular genetic approaches of restriction enzyme mediated integration (Shim and Woloshuk, 1999) and targeted gene disruption (Proctor *et al.*, 1999). In the current study, we have shown that it is also possible to generate fumonisin biosynthetic mutants via UV mutagenesis. The results of genetic analyses of strains with the UV-induced mutation support the previously reported (Desjardins *et al.*, 1996a) linkage between *Fum2* and *Fum3* and provide further evidence for a fumonisin biosynthetic gene cluster. In addition, the use of the RAPD makers in the analysis facilitated mapping of the *Fum* loci along a linkage group of *G. fujikuroi* (Figure 3). Xu and Leslie (1996) demonstrated that *Fum1* maps to a position on linkage group 1 between RFLP markers 5E50 and 78E. In other studies, Desjardins and coworkers mapped the positions of RAPD markers OPA16 and OPH3 relative to *Fum1* (Desjardins *et al.*, 1996b) and to the RFLP markers 5E50 and 78E (Proctor *et al.*, unpublished data) and demonstrated that *Fum1*, *Fum2* and *Fum3* are linked to one another (Desjardins *et al.*, 1996b). In the current study, we mapped the positions of *Fum2* and *Fum3* relative to the RAPD markers. Together, these data reveal the orientation of the *Fum* loci and the RAPD and RFLP markers along linkage group 1 of *G. fujikuroi* MP-A and provide a more detailed genetic map of this region than has previously been reported (Figure 3).

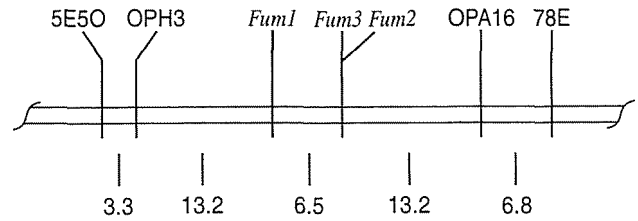

Figure 3. Linkage map of *Fum* loci, RAPD markers OPA16 and OPH3, and RFLP markers 5E50 and 78E on linkage group 1 of *G. fujikuroi* MP-A. Xu and Leslie (1996) identified the RFLP markers on linkage group 1 and determined their positions relative to *Fum1*, Desjardins *et al.* (1996a) determined the recombination frequency between *Fum1*, *Fum2* and *Fum3*, and Proctor *et al.* (unpublished data) determined the recombination frequencies between 5E50, OPH3, 78E, and OPA16

The *Fum2* and *Fum3* loci confer the ability to hydroxylate the fumonisin backbone at C-10 and C-5, respectively, and as a result are thought to encode hydroxylases (Desjardins *et al.*, 1996a). The results from the current study suggest either that the putative hydroxylases had markedly different substrate specificities or that exogenously added FB_2, FB_3 and FB_4 were differentially excluded from the cellular locations where C-5 and C-10 hydroxylations occur. C-5 hydroxylation of exogenously added FB_3 and FB_4 occurred readily in strains with a wild-type *Fum3* locus (Table 3), suggesting that both fumonisins are good substrates for the putative *Fum3* hydroxylase and that neither is excluded from the cellular location where C-5 hydroxylation occurs. In contrast, C-10 hydroxylation of exogenously added FB_2 did not occur and occurred only poorly for FB_4 in cultures of strains with a wild-type *Fum2* locus (Table 3). The isolation and characterization of the genes at the *Fum2* and *Fum3* loci may provide tools that can be used to determine whether the lack of and poor hydroxylation at C-10 resulted from substrate specificity, site exclusion or both. Since the putative *Fum2* hydroxylase appears to act primarily before the formation of FB_3, the inactivation of

the gene(s) at this locus may lead to the accumulation of an as yet unidentified biosynthetic intermediate and provide further information on the point at which the fumonisin pathway branches. In addition, if fumonisin biosynthetic genes are indeed clustered, genes encoding enzymes that catalyse the carbonyl reduction, methylation, alanine condensation and esterification of the backbone should be located near genes at the *Fum2* or *Fum3* loci or near the fumonisin polyketide synthase gene (*FUM5*) that has already been isolated.

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